An Environmentally-Controlled Microplate Reader with Injector Enables Walk-Away Monitoring of Cytotoxicity, Viability and Apoptosis

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1. Introduction

Assaying cells in an appropriate window of time after a compound-mediated cytotoxic event can be extremely challenging. Missing this window may mean the difference between defining an effect as apoptotic or necrotic and can affect interpretation of a compound's mode of action. Monitoring membrane integrity changes and cell viability can reveal an exact point in time when primary or secondary necrotic events occur. Further multiplexing with a caspase-3/7 assay can help differentiate membrane breakdown due to apoptosis and secondary necrosis from an overt cytotoxic event.

Here we describe a method for kinetic monitoring of cytotoxicity and viability. We evaluate apoptosis using the Tecan Spark™ 10M to inject caspase-3/7 reagent at timed intervals. Further studies investigate the ability of the Spark to automatically inject caspase-3/7 reagent using a cytotoxicity threshold set within the reader software. We evaluate the effects of compounds, including six kinase inhibitors, on K562 cell health over time.

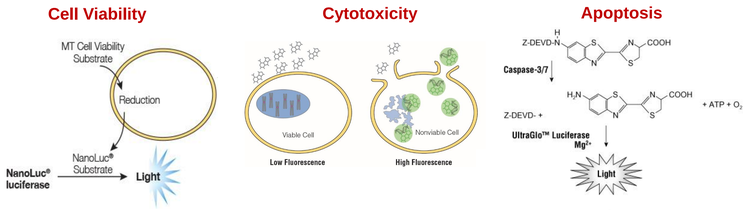
2. The Tecan Spark™ 10M Microplate Reader

Figure 1. The Spark™ 10M with reagent injector.

Table 1. Assay requirements and the corresponding Spark feature used.

Assay Requirement	Spark Feature Used
Incubate 384 well assay plate	Temperature control (37°C)
Maintain 5% CO_2 and ambient O_2	Gas control
Prevent medium evaporation	Humidity cassette with liquid reservoir
Quantify cell viability	Lid lifter, top-read luminescence
Quantify cytotoxicity	Monochromator, bottom-read fluorescence (EX 485/20, EM 520/20)
Add caspase-3/7 reagent	Lid lifter, reagent injector
Quantify apoptosis	Lid lifter, top-read luminescence

3. Viability, Cytotoxicity, and Apoptosis Assays Can Be Multiplexed Together in the Same Well



RealTime-Glo™ MT Cell Viability Assay
- Non-lytic, kinetic
- Viable cells reduce pro-NanoLuc® substrate
- NanoLuc® luciferase uses NanoLuc® substrate to produce light
- Luminescence is proportional to number of viable cells

CellTox™ Green Cytotoxicity Assay
- Non-lytic, kinetic
- Asymmetric cyanine dye binds DNA released from dead cells
- Dye becomes fluorescent when bound to DNA
- Fluorescence is proportional to number of dead cells

Caspase-Glo® 3/7 Assay
- Lytic, endpoint
- Caspase-3/7 cleaves pro-luminescent DEVD-luciferin substrate.
- Firefly luciferase uses luciferin substrate to produce light
- Luminescence is proportional to caspase-3/7 activity (apoptosis)

4. A Simple Workflow for Walk-Away Data Generation

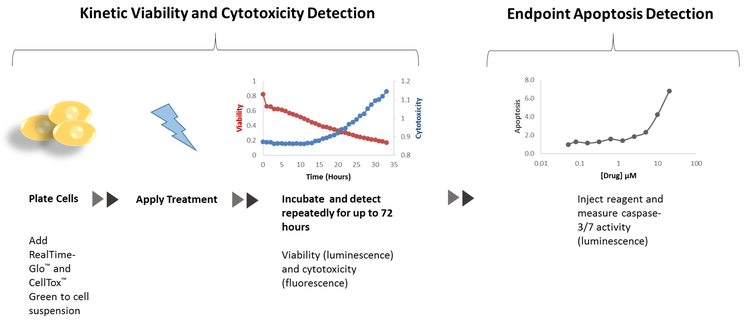


Figure 2. Walk-away data generation is achieved following plate setup. Viability and cytotoxicity are monitored in real-time. Caspase-Glo® 3/7 reagent addition is dictated by the user and added at specific intervals, or if certain experimental conditions are met.

5. Monitoring Effects of Ponatinib on K562 Cell Health Using Timed Caspase-3/7 Reagent Injection

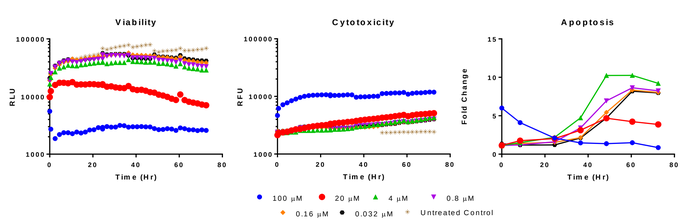
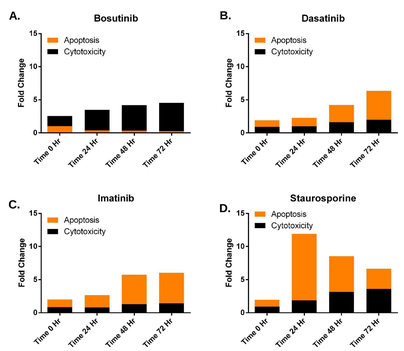


Figure 3. Time and dose-dependent effects on K562 cell health following Ponatinib treatment. Viability and cytotoxicity were monitored every hour. Caspase-Glo® 3/7 reagent was injected at time 0 and every 12 hours. Freshly prepared Caspase-Glo® 3/7 Reagent was added to the instrument every 24 hours. Immediate cytotoxicity with moderate caspase induction is noted at high concentration (100µM). Anti-proliferative effects with moderate caspase induction are noted at mid-range concentration (20µM). Longer term exposure to lower concentrations (≤ 4µM) past 40 hours reveals the most potent caspase induction with decreases in cell viability and increases in cytotoxicity beginning to emerge.

6. The Detection of Caspase-3/7 Activity is Correlated with K562 Cytotoxicity and Is Transient in Nature



All kinase inhibitors show an increase in cytotoxicity over time due to treatment with 100µM drug. Apoptosis (caspase-3/7 activity) is generally least detectable in cases of early cytotoxicity (A). Apoptosis detection increases with time and is correlated with an increase in cytotoxicity (B-D).

The transient nature of caspase-3/7 detection is noted in the case of Bosutinib (A) and Staurosporine (D) where the level of caspase-3/7 detection decreases with time.

Figure 4. Fold changes in cytotoxicity and apoptosis as compared to an untreated control reveal varied cell health profiles in response to kinase inhibitor treatment.

7. Using the Cytotoxicity Readout to Initiate Automated Caspase-3/7 Reagent Injection

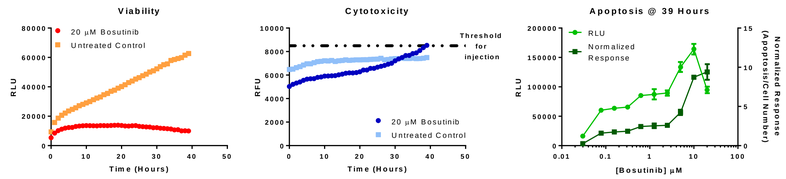


Figure 5. The cytotoxicity measurement can be used to initiate automated injection of caspase reagent. All wells within the dose range of Bosutinib were monitored in real time for viability and cytotoxicity. A fluorescence threshold was used to initiate injection of caspase reagent. When the fluorescence threshold value of the highest compound concentration (20µM) was achieved, reagent was injected to all wells in the dose series, the plate was incubated for 30 minutes, and luminescence from the apoptosis assay was quantified.

Caspase-3/7 activity was detectable at 39 hours across the entire dose series of Bosutinib indicating potent induction of apoptosis compared to the control. The viability assay shows that the highest concentration of Bosutinib exhibits anti-proliferative effects within 10 hours of treatment. As a result, a decrease in caspase-3/7 activity at 39 hours is likely due to the decreased number of K562 cells present in the well at the time of reagent injection. Normalizing apoptosis to cell number (viability) accounts for this anti-proliferative effect.

8. Automated Caspase-3/7 Reagent Injection Confirms Mechanism of Action

Table 2. Mechanism of action is confirmed for all compounds tested. Staurosporine and bcr/abl-targeted inhibitors, with the exception of Ponatinib, show detectable apoptosis at the time of reagent injection. Ponatinib results are in accordance with those obtained in panel 5; at 14 hours incubation only cytotoxicity is detectable, and with increased incubation apoptosis is likely to be measured. Ionomycin, known to cause cytotoxicity and necrosis, shows no apoptosis at 5 hours.

Compound [20µM]	Mechanism of Action	Time of Injection	Apoptosis (Fold Change over untreated control)	Cytotoxicity (Fold Change over untreated control)	Viability (Fold Change over untreated control)
Ionomycin	Calcium ionophore	5 Hours	1.1	1.15	0.61
Ponatinib	Bcr/abl kinase inhibitor	14 Hours	1.2	1.14	0.14
Staurosporine	Pan-kinase inhibitor	33 Hours	6.8	1.14	0.17
Imatinib	Bcr/abl kinase inhibitor	35 Hours	12.0	1.15	0.32
Bosutinib	Bcr/abl kinase inhibitor	39 Hours	5.8	1.09	0.16
Dasatinib	Bcr/abl kinase inhibitor	53 Hours	7.2	1.14	0.36

9. Conclusions

Promega Cell Health Assays and the Tecan Spark™ 10M support mechanistic toxicity determination with data acquisition for multiple days
- Viability and cytotoxicity can be monitored in real-time up to 72 hours
- The lid lifter provides access to the assay plate for data acquisition and timed or automatic reagent injection

Membrane integrity changes correlate with the level of apoptosis detected
- Early detection of cytotoxicity correlates with fast-acting apoptosis inducers or overtly cytotoxic compounds (1° necrosis)
- Delayed detection of cytotoxicity correlates with more potent induction of apoptosis (2° necrosis)

Cytotoxicity measurement can dictate the time of caspase-3/7 reagent injection
- Caspase-3/7 activity at cytotoxic threshold values correlated with compounds known to induce apoptosis